The Dynamics of Protein Kinase B Regulation during B Cell Antigen Receptor Engagement

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Abstract. This study has used biochemistry and real time confocal imaging of green fluorescent protein (GFP)-tagged molecules in live cells to explore the dynamics of protein kinase B (PKB) regulation during B lymphocyte activation. The data show that triggering of the B cell antigen receptor (BCR) induces a transient membrane localization of PKB but a sustained activation of the enzyme; active PKB is found in the cytosol and nuclei of activated B cells. Hence, PKB has three potential sites of action in B lymphocytes; transiently after BCR triggering PKB can phosphorylate plasma membrane localized targets, whereas during the sustained B cell response to antigen, PKB acts in the nucleus and the cytosol. Membrane translocation of PKB and subsequent PKB activation are dependent on BCR activation of phosphatidylinositol 3-kinase (PI3K). Moreover, PI3K signals are both necessary and sufficient for sustained activation of PKB in B lymphocytes.

However, under conditions of continuous PI3K activation or BCR triggering there is only transient recruitment of PKB to the plasma membrane, indicating that there must be a molecular mechanism to dissociate PKB from sites of PI3K activity in B cells. The inhibitory Fc receptor, the FcγRIIB, mediates vital homeostatic control of B cell function by recruiting an inositol 5 phosphatase SHIP into the BCR complex. Herein we show that coligation of the BCR with the inhibitory FcγRIIB prevents membrane targeting of PKB. The FcγRIIB can thus antagonize BCR signals for PKB localization and prevent BCR stimulation of PKB activity which demonstrates the mechanism for the inhibitory action of the FcγRIIB on the BCR/PKB response.

Key words: Akt/PKB • phosphatidylinositol 3-kinase • GSK3 • B cell antigen receptor • FcγRIIb

FOREIGN antigen binding to the B cell antigen receptor (BCR)[1] triggers the activation of cytosolic tyrosine kinases including Syk and lyn and BTK (Yamanashi et al., 1992; Desiderio and Siliciano, 1994; Rawlings et al., 1996; DeFranco, 1997). These tyrosine kinases then initiate a cascade of signaling pathways that control B cell function during an immune response. The BCR regulates the metabolism of inositol phospholipids by two pathways: BCR stimulation of phospholipase C results in the hydrolysis of phosphatidylinositol (4,5) biphosphate [PI(4,5)P$_2$] and the production of inositol 1,4,5-triphosphate which initiates an increase in intracellular calcium (Cambier and Jensen, 1994). PI(4,5)P$_2$ breakdown simultaneously produces diacylglycerol which activates serine/threonine kinases of the protein kinase C family. BCR triggering also stimulates the activity of phosphatidylinositol 3-kinase (PI3K) which phosphorylates PI(4,5)P$_2$ on the D-3 position of the inositol ring to produce PI(3,4,5)P$_3$ (Gold et al., 1992). D-3 phosphoinositides bind to the plextrin homology (PH) domains of proteins and either allosterically modify their activity or induce relocalization of the protein to defined areas of the plasma membrane where activation can occur (Rameh et al., 1997). Genetic evidence for the importance of PI3K for B lymphocytes has been illustrated by the phenotype of mice lacking expression of the p85α regulatory adapter protein for PI3K which show profound defects in B cell function (Fruman et al., 1999; Suzuki et al., 1999).

During B cell activation both positive and negative regulatory signaling cascades are vital for a balanced immune response and immune homeostasis. One important negative feedback mechanism that operates in B cells is mediated by the FcγRIIb and the src homology 2 (SH2) domain containing inositol 5′ phosphatase (SHIP) (Takai et al., 1996; Ono et al., 1997; Tridandapani et al., 1997; Helgason et al., 1998; Okada et al., 1998; Sarao et al.,

1. *Abbreviations used in this paper:* BCR, B cell antigen receptor; GFP, green fluorescent protein; GSK3, glycogen synthase kinase 3; PH, plextrin homology; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B.

1998). Coligation of the BCR with the FcγRIIB by antigen–antibody complexes results in tyrosine phosphorylation of an immune cell tyrosine based inhibitory motif (ITIM) in the FcγRIIB (Burshtyn and Long, 1997). The SH2 domain of SHIP can bind to the phosphorylated ITIM, thereby recruiting this inositol 5′ phosphatase into the BCR/FcγRIIB complex. SHIP dephosphorylates PI $(3,4,5)P_3$ to produce $PI(3,4)P_2$ and accordingly, coligation of the BCR with FcγRIIB diminishes BCR elevation of intracellular $PI(3,4,5)P_3$. PI3K controlled signaling pathways are thus the proposed primary targets for FcγRIIb inhibition (Hippen et al., 1997; Scharenberg et al., 1998; Scharenberg and Kinet, 1998).

Recent studies have identified two proteins regulated by PI3K and SHIP in B cells: a tyrosine kinase Btk (Bolland et al., 1998; Scharenberg et al., 1998) and a serine/threonine kinase protein kinase B or Akt/PKB (Aman et al., 1998; Jacob et al., 1999), the cellular homologue of the transforming oncogene of the murine retrovirus Akt8 (Staal and Hartley, 1988). PKB has been shown to play an important role in maintenance of cell survival in fibroblasts and epithelial cells (Zha et al., 1996; Kauffmann-Zeh et al., 1997; Kulik et al., 1997). In T cells, PKB can stimulate the activity of E2F transcription factors which are important components of the mechanisms that control the mammalian cell cycle (Brennan et al., 1997). PKB also phosphorylates and inactivates GSK3 (Cross et al., 1995), an enzyme initially identified as a regulator of glycogen metabolism but which also has broader functions: interactions between GSK3 and β-catenin (Rubinfeld et al., 1996) regulate activity of the T cell factor–lymphoid enhancer factor (Tcf-Lef). GSK3 can also control the nuclear export of nuclear factor of activated T cells (NFAT), transcription factors involved in cytokine gene induction (Beals et al., 1997).

In this report we have explored the dynamics of PKB regulation by the BCR and the inhibitory FcγRIIB in B lymphocytes. We used green fluorescent protein (GFP; Heim and Tsien, 1996; Rizzuto et al., 1996) to tag PKB and time lapse confocal microscopy of live cells to reveal that BCR regulation of Akt/PKB has the following sequence: a rapid and transient recruitment of Akt/PKB to the plasma membrane followed by a sustained activation of the enzyme. Analysis of the location of GFP-tagged PKB indicated that active PKB is not normally maintained at the plasma membrane but is found in the cytosol and nucleus. We show that PI3K signals are both sufficient for membrane recruitment of Akt/PKB and for sustained activation of the enzyme. BCR activation of PKB is subject to a negative feedback control mechanism initiated by the FcγRIIB and mediated by SHIP (Aman et al., 1998). We show that coligation of the BCR with the inhibitory FcγRIIB prevents membrane localization of PKB. These data illustrate the importance of PI3K-mediated signals in controlling the cellular localization of PKB and they reveal the mechanism for the inhibitory action of the FcγRIIB on the BCR/PKB response.

Materials and Methods

Cells, Antibodies, Constructs, and Reagents

The BALB/c mouse B lymphoma line, A20, was cultured in RPMI 1640 supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. Rabbit anti–mouse whole Ig antibody and F(ab′)$_2$ fragment were obtained from Zymed. The serine 473 phosphospecific PKB antibody was purchased from New England Biolabs, the phosphospecific GSK3α and the pan anti-PKBα were from Upstate Biotechnology, the pan anti-GSK3α antisera were from Santa Cruz Biotechnology. Rabbit antisera reactive with the COOH-terminal residues 904–918 of PKD/PKCμ were generated by standard protocols. Anti–sheep and anti–goat HRP-conjugated antibodies were obtained from Chemicon, and anti–mouse HRP and anti–rabbit HRP were from Sigma Chemical Co. The rat CD2 monoclonal antibody OX34 and the 12CA5 monoclonal reactive with the Ha epitope tag were purified from hybridoma supernatants by standard protocols. The FcγRIIB blocking antibody (FcBlock™) was purchased from PharMingen. The PI3K inhibitor LY294002 was purchased from BIOMOL Research Laboratories, the histone H2B was from Boehringer Mannheim, the protein kinase inhibitor and ATP were from Sigma Chemical Co., and the $[\gamma\text{-}^{32}P]ATP$ was from Amersham.

A chimeric protein of the extracellular and transmembrane domain of the rat CD2 molecule fused with the p110a catalytic subunit of PI3K (rCD2p110) targets PI3K to the membrane and creates a constitutively active enzyme that generates $PI(3,4,5)P_3$ and $PI(3,4)P_2$. The rCD2p110 construct and a catalytically inactive form with a mutated ATP-binding site (rCD2-p110R/P) were described previously (Reif et al., 1996, 1997). The GFP-tagged PH domain of PKB and its non–lipid-binding R25C mutated form were generated, respectively, from subcloning HindIII/BamH1 from pCMV6 containing HA-tagged murine PKBα PH domain (HA-AH) or HA-R25C (Franke et al., 1995) into -C1 (Clontech), the BsrG1/Sal1 fragment was then replaced by a linker of six glycine residues. Full length PKB was COOH-terminally GFP-tagged using standard protocols. Oligonucleotides introduced in pEGFP (Clontech) a sequence corresponding to the COOH-terminal sequence of PKB from the BclI site to the end of the protein at the NH$_2$ terminus of GFP and a BglII site and a stop codon at the COOH terminus of GFP. This resultant fragment was purified and ligated to pSG5 HA-PKB digested with BclI and BglII. All constructs were verified by DNA sequencing. The GFP-PKB fusion protein undergoes activation in response to BCR ligation (data not shown).

Cell Stimulation and Transfection

A20 cells were washed and resuspended at 2×10^7/ml in RPMI 1640. The cells were incubated at 37°C with either 10 μg/ml of anti–mouse F(ab′)$_2$ fragment or 15 μg/ml of intact Ig, for the indicated periods of time. Some samples were preincubated with 5 μM of Ly294002 or 2.5 μg/ml anti-FcγRIIb for 30 min before stimulation. Cells were quickly pelleted at 4°C and lysed in 50 mM Hepes, 10 mM NaF, 10 mM iodoacetamide, 75 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM Na$_2$VO$_3$, and 1 μg/ml of each for leupeptin, pepstatin A, and chymotrypsin. Nuclear debris were eliminated by 20 min centrifugation at 14,000 rpm and the supernatant proteins precipitated for 1 h at −70°C in 1.5 vol acetone. Precipitates were pelleted by 20 min of centrifugation at 14,000 rpm, resuspended in Laemmli buffer, boiled, and fractionated on 10% SDS-PAGE, with the exception of samples for GSK3 blotting, which were analyzed on 7.5% gels. Immunoblotting was performed by standard protocols and revealed by chemiluminescence (ECL; Amersham). For transfection, 500 μl of cells was aliquoted with DNA, electroporated at 310 V and 960 μF using a Bio-Rad gene pulser. Thereafter cells were cultured for 12–14 h before stimulation, microscopic analysis, or lysis.

Immunoprecipitation and Kinase Assay

5×10^6 cells transfected with HA-tagged PKB were stimulated as indicated and lysed in 120 mM NaCl, 50 mM Hepes, 10 mM NaF, 1 mM EDTA, 40 mM β-glycerophosphate, 1% NP-40, 1 mM Na$_2$VO$_3$, and 1 μg/ml of each for leupeptin, pepstatin A, and chymotrypsin. After elimination of nuclear debris, the supernatant was cleared by addition of 1/25 vol of a 30% solution of Sepharose-coupled protein G. Supernatants were incubated for 2 h with 10 μg/ml of the anti-HA antibody 12CA5, and another 45 min after addition of 1/10 vol of the Sepharose–protein G solution. Pellets were washed once in lysis buffer, twice in 500 mM LiCl, 100 mM Tris, pH 7.5, 1 mM EDTA, and once in assay buffer (50 mM Tris, pH 7.5, 10 mM MgCl$_2$, and 1 mM DTT). Dried samples were assayed by incubation for 30 min at room temperature with 2.5 μg H2B, 5 μM protein kinase inhibitor, 50 μM ATP, and 3 μCi of $[\gamma\text{-}^{32}P]ATP$ in a final volume of 15 μl. Boiled samples in Laemmli buffer were fractionated by SDS-PAGE. The level of H2B phosphorylation was analyzed by exposing the lower part of the gel

on a sensitive film, and the amount of PKB protein immunoprecipitated was determined by Western blot analysis with a pan PKB antibody.

Nuclear Fractionation

After stimulation, cells were disrupted in 10 mM Hepes, 15 mM KCl, 2 mM MgCl$_2$, 0.1 mM EDTA, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM Na$_2$VO$_3$, 0.15% NP-40, and the nuclear fraction pelleted at 14,000 rpm for 1 min. Cytosolic extracts were removed and NaCl was added to obtain a final concentration of 120 mM. These cytosolic extracts were then concentrated by acetone precipitation. The nuclear pellets were washed three times with lysis buffer and then resuspended in 20 mM Hepes, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 20% glycerol, 0.42 M NaCl, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM Na$_2$VO$_3$, 0.15% NP-40, and lysed for 30 min at 4°C. Debris was eliminated by 20 min of centrifugation at 14,000 rpm and supernatants, corresponding to nuclear extracts, were acetone precipitated.

Confocal Time Lapse Imaging

After transfection, cells were resuspended at 10^6/ml in 35-mm glass bottom dishes (MatTek Corp.). Before analysis dishes were gently rinsed with warm Hanks' medium without phenol red. Dishes were mounted in the warm chamber of the confocal microscope (Zeiss Laser Scanning Microscope 5.10). Samples were excited at 488 nm by an argon laser and detected with a 63 × 1.4 NA oil immersion objective. The first image was recorded just before stimulus addition directly into the dish, and then scans were made automatically every 5 s by using Zeiss LSM software. Images shown are representative of a minimum of five experiments. In Fig. 2, anti-FcγRIIB and LY294002 were added to the dishes in Hanks' medium 30 min before microscopic analysis.

Results

The B lymphoma cell line A20 was either left unstimulated or activated by cross-linking the BCR with F(ab')$_2$ fragment of anti–mouse IgG. Active PKB is phosphorylated on two residues; threonine 308 and serine 473 by PI3K-dependent protein kinases (Alessi et al., 1997; Alessi and Cohen, 1998; Bellacosa et al., 1998; Stephens et al., 1998). To monitor PKB activity in cells, total cell extracts were prepared and fractionated by SDS-PAGE and processed for Western blot analysis with a specific antisera that recognizes active PKB molecules phosphorylated on serine 473. The phospho-PKB antisera did not react with PKB present in cell lysates from quiescent cells, whereas in cells activated by cross-linking the BCR with F(ab')$_2$ fragment of anti–mouse IgG there was a strong reactivity of PKB with the phospho-PKB antisera (Fig. 1). The data in Fig. 1 B show the in vitro catalytic activity of immune complexes of PKB isolated from quiescent or BCR-triggered cells, assayed using histone H2B as a substrate. These data confirm that stimulation of B cells via the BCR activates the catalytic activity of PKB. This result was confirmed also by analysis of the effects of BCR ligation on the phosphorylation of GSK3, an endogenous substrate for PKB (Cross et al., 1995). Phosphospecific antisera with selectivity for GSK3 molecules phosphorylated on the PKB substrate sequence do not recognize GSK3 proteins isolated from quiescent B cells but are strongly reactive with the GSK3 present in cell lysates prepared from BCR-triggered cells (Fig. 1 C).

Cross-linking of the BCR with the FcγRIIB inhibits BCR signal transduction pathways by recruiting the inositol 5′ phosphatase SHIP into the BCR complex (Ono et al., 1997; Tridandapani et al., 1997). The data in Fig. 1 A show that B cell activation with intact IgG, which cross-links the
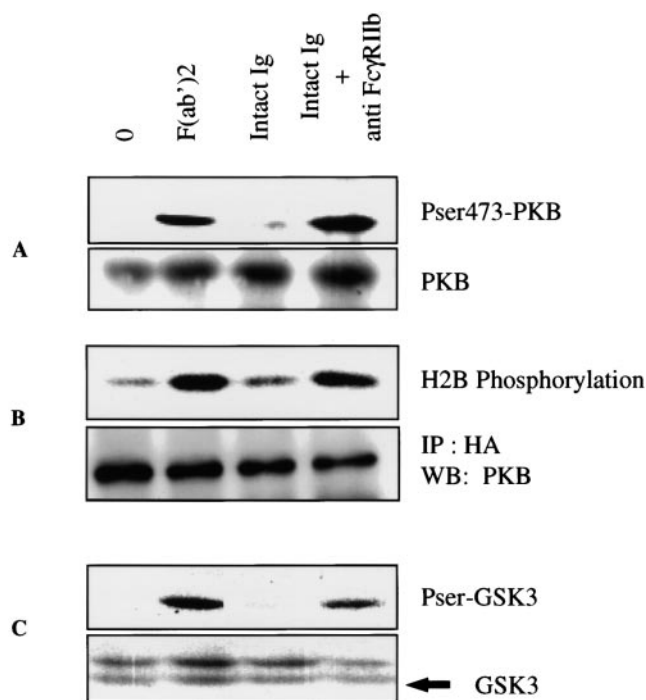

Figure 1. BCR and FcγRIIb regulation of PKB. The data show PKB phosphorylation and activity in quiescent A20 cells or A20 cells stimulated for 10 min at 37°C with the following stimuli: 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR; 15 μg/ml of intact IgG of anti–mouse IgG which coligates the BCR and the FcγRIIB complex; 15 μg/ml of intact Ig in cells pretreated for 30 min with 2.5 μg/ml anti-FcγRIIB which prevents FcγRIIB/BCR coligation and allows intact Ig to trigger the BCR. The data in A show Western blots of total cell lysates performed with serine 473 phosphospecific PKB antibody and pan PKB antisera. The data in B show PKB kinase activity of immunoprecipitated Ha epitope–tagged PKB measured using histone H2B as a substrate. H2B substrate phosphorylation was analyzed by autoradiography. The data in C show Western blots of total cell lysates performed with phosphospecific GSK3α and pan GSK3α antisera.

BCR with the FcγRIIB, fails to stimulate PKB phosphorylation or PKB catalytic activity as judged by in vitro (Fig. 1 B) and in vivo (Fig. 1 C) assays of PKB function. In the presence of an anti-FcγRIIB antibody which blocks the interaction of IgG with the FcγRIIB receptor, intact IgG is able to trigger the BCR without coligating inhibitory FcγRIIB into the BCR complex. The presence of the blocking antibody to the FcγRIIB then allows intact IgG to ligate the BCR and stimulate PKB activity (Fig. 1).

PKB Localization in B Lymphocytes

Membrane localization of PKB is known to be essential for the activation of the enzyme but there are conflicting reports as to whether activated PKB is present in the nucleus or maintained at the cell membrane (Andjelkovic et al., 1997; Meier et al., 1997; Watton and Downward, 1999). To examine the localization of PKB carefully in intact B cells we expressed a GFP-tagged construct of wild-type PKB in A20 cells and analyzed the cellular localization of this enzyme in quiescent and BCR-activated cells. Membrane
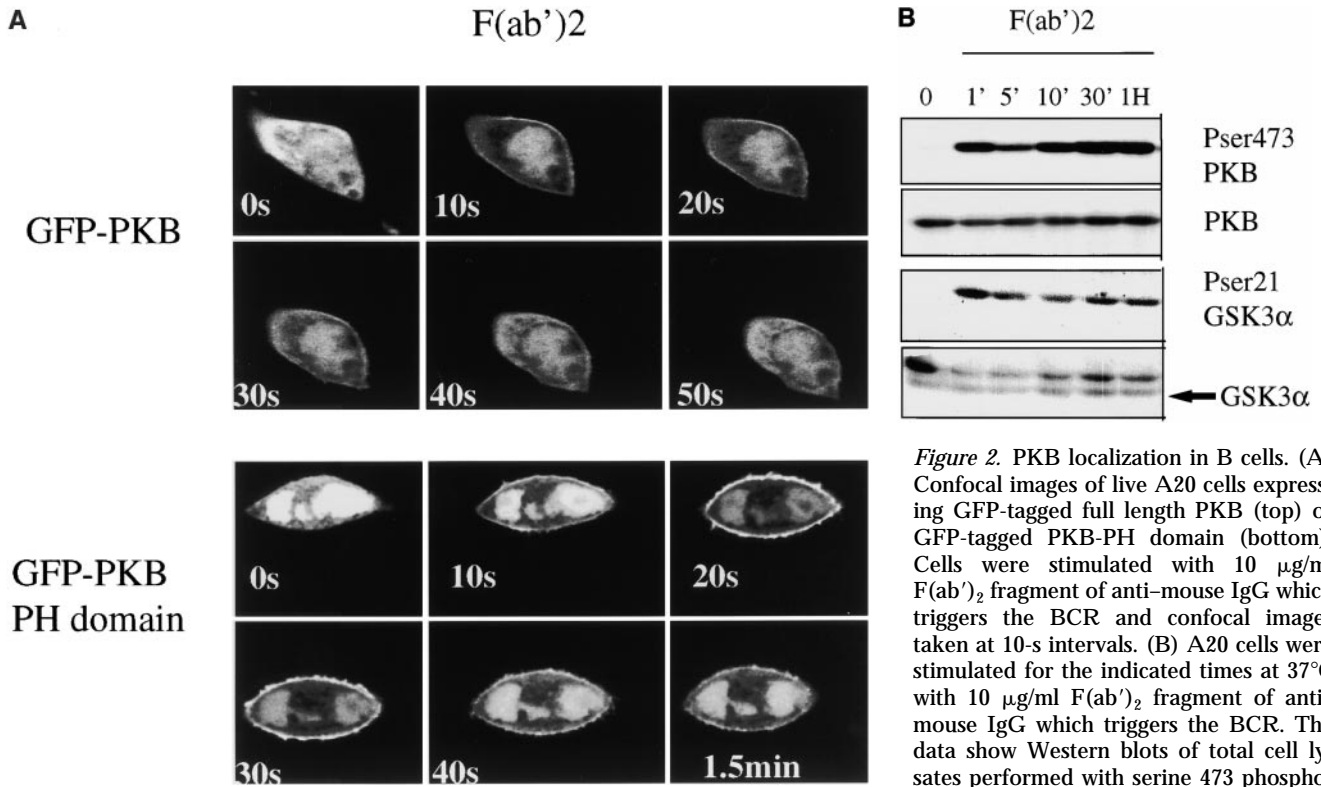
A

F(ab')2

GFP-PKB

GFP-PKB
PH domain

B

F(ab')2

0 1' 5' 10' 30' 1H

Pser473 PKB

PKB

Pser21 GSK3α

← GSK3α

Figure 2. PKB localization in B cells. (A) Confocal images of live A20 cells expressing GFP-tagged full length PKB (top) or GFP-tagged PKB-PH domain (bottom). Cells were stimulated with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR and confocal images taken at 10-s intervals. (B) A20 cells were stimulated for the indicated times at 37°C with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR. The data show Western blots of total cell lysates performed with serine 473 phospho-specific PKB antibody and pan PKB antisera and phosphospecific GSK3α and pan GSK3α antisera.

targeting of PKB is mediated by interactions of the PH domain with either PI(3,4,5)P$_3$ or PI(3,4)P$_2$. Accordingly, we examined also the cellular localization of a GFP-tagged PKB-PH domain. Fig. 2 shows midsection confocal images of A20 cells transiently transfected with GFP wild-type PKB or the GFP-tagged PKB-PH domain. In unstimulated A20 cells, GFP-PKB was uniformly distributed throughout the cytosol of the cell and was also present in the nucleus. The GFP-tagged PKB-PH domain of PKB was similarly distributed. Western blot analysis of nuclear extracts prepared from A20 cells revealed that endogenous PKB was present in the nuclei of these lymphocytes (Fig. 3).

BCR Ligation Induces a Transient Relocalization of PKB to the Plasma Membrane but a Sustained Activation of the Enzyme

A20 cells expressing GFP-PKB constructs were stimulated with F(ab')$_2$ fragment of anti–mouse IgG and confocal images of a midsection of the cells were taken at 10-s intervals after BCR ligation. This allows the effects of BCR triggering on the cellular localization of PKB to be monitored in live cells in real time. These images in Fig. 2 show that triggering of the BCR induces a rapid membrane localization of both full length PKB and the PKB-PH domain. The membrane localization of both PKB constructs was detected within 10 s of BCR ligation. Consistently, it was seen that wild-type PKB recycles from the membrane within 40–60 s of BCR triggering. In contrast, the translo-

cation of the PKB-PH domain to the membrane was sustained (Fig. 2 A). The motility of the activated B cells makes longer periods of confocal imaging live cells difficult but there was no indication of recycling of the PKB-
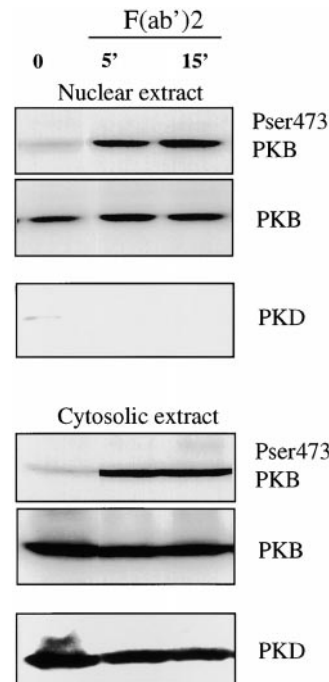

F(ab')2

0 5' 15'

Nuclear extract

Pser473 PKB

PKB

PKD

Cytosolic extract

Pser473 PKB

PKB

PKD

Figure 3. Cellular localization of PKB in B cells. The data show Western blot analyses of nuclear and cytosolic extracts of A20 cells performed with serine 473 phosphospecific PKB antibody and pan PKB antisera or an antisera reactive with the serine/threonine kinase PKD/PKCμ. Cells were either quiescent or stimulated for the indicated times with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG.

PH domain away from the membrane over a period of minutes. Thus, BCR-induced translocation of the PKB-PH domain to the plasma membrane is stable compared with the response of the full length GFP-tagged wild-type PKB.

A striking feature of the effect of the BCR on PKB membrane localization is its speed and transience. Kinetic analysis of the effect of the BCR on PKB activity reveals that this is also a rapid response detectable within 30 s of BCR triggering (data not shown). The data in Fig. 2 B show that the effect of BCR ligation is to induce a stable activation of PKB in a response that is maximal at 1 min and sustained for at least 60 min (Fig. 2 B). It is also clear that BCR induced phosphorylation of GSK3; an endogenous substrate for PKB is a response that is maintained for a period of 1–2 h (Fig. 2 B). In summary, analysis of PKB cellular localization and activity shows that quiescent B cells express PKB in the cytosol and the nucleus. BCR triggering induces rapid membrane localization and activation of PKB. PKB targeting to the membrane in response to BCR ligation is very transient and can be detected within 10 s but is finished by 40–50 s. In contrast, the stimulatory effects of the BCR on PKB activity are sustained for at least 1 h. 1 min after BCR triggering there is no discernible plasma membrane localized PKB, rather the active PKB is present in either the cytosol or the nucleus.

Active PKB Is Found in the Nucleus and Cytosol of BCR-triggered Cells

The question of the localization of active PKB is important because it affords insight as to the potential site of action of this kinase. To determine whether active PKB is present in the nuclear or cytosolic compartment of B cells, A20 cells were triggered via the BCR and cytosolic and nuclear cell fractions were resolved by SDS-PAGE and analyzed by Western blot with the phospho-PKB antibody that recognizes active enzyme or with the pan PKB antisera. These data (Fig. 3) show that endogenous PKB is found in both the nucleus and cytosol of quiescent or activated B cells. In cells activated by cross-linking the BCR with $F(ab')_2$ fragment of anti–mouse IgG there was a strong reactivity of both cytosolic and nuclear PKB with the phospho-PKB antisera. These results show that active PKB is found in both the cytosol and nucleus of activated B lymphocytes.

PI3 Kinase Signals Are Essential and Sufficient for Membrane Localization and Activation of PKB in B Cells

PI3K signals are essential and sufficient for activation of PKB in T lymphocytes (Reif et al., 1997). A comparison of the data in Fig. 4, A and B, shows that the BCR-induced transient membrane localization of GFP-PKB is abolished by the PI3K inhibitor Ly294002. The data in Fig. 4 C show that Ly294002 also prevents BCR activation of PKB. Hence, the ability of the BCR to regulate the cell localization and activity of PKB is dependent on PI3 kinase activity. To determine whether PI3K signals are sufficient for membrane targeting and activation of PKB we examined the effects of expression of constitutively active PI3 kinase on PKB cellular localization and activity. The BCR stimu-

lates the activity of a PI3K complex that comprises a regulatory p85 and a catalytic p110 subunit. A chimeric protein of the extracellular and transmembrane domain of the rat CD2 molecule fused with the p110α catalytic subunit of PI3K (rCD2p110) targets PI3K to the membrane and creates a constitutively active enzyme that generates $PI(3,4,5)P_3$ and $PI(3,4)P_2$ when expressed in cells. The data in Fig. 5 show that expression of the constitutively active PI3K, rCD2p110, induces a strong activation of PKB. In these experiments A20 cells were transfected with increasing concentrations of the rCD2p110 expression vector resulting in increasing levels of expression of rCD2p110. The effect of the active rCD2p110 constructs on endogenous PKB activity was monitored using the phospho-PKB antisera. The results (Fig. 5 A) show that there was a dose-dependent stimulation of PKB activity by the expressed active PI3K. This stimulatory effect of rCD2p110 was dependent on the kinase activity of the chimera since expression of a kinase inactive mutant, rCD2p110 R/P, did not stimulate PKB activity. Moreover, treatment of A20 cells with the PI3K inhibitor LY294002 abrogated PKB activity in cells expressing rCD2p110 (Fig. 5 B).

Confocal microscopy of cells cotransfected with rCD2-p110 and GFP-tagged PKB constructs shows the localization of PKB in cells expressing constitutively active PI3K. These data reveal that membrane-localized PKB can be detected in cells expressing active PI3 kinase (Fig. 6 A). However, these cells also contain significant levels of cytosolic and nuclear GFP-PKB. In marked contrast, the total cellular pool of the GFP-tagged PH domain of PKB is constitutively present at the membrane of cells expressing active PI3K (Fig. 6, A and B). In A20 cells stimulated via the BCR (Fig. 2) we had noted that the wild-type PKB is only transiently present at the cell membrane, whereas the BCR-induced translocation of the isolated GFP-tagged PKB-PH domain to the plasma membrane was sustained. The results in Fig. 6 show clearly that in cells expressing a constitutively active PI3K there is a stable and sustained plasma membrane localization of the total cellular pool of the PKB-PH domain but not a sustained membrane recruitment of intact PKB.

The ability of active PI3K to sustain membrane localization of the PKB-PH domain is dependent on the catalytic activity of the enzyme. The application of the PI3K inhibitor, Ly294002, to cells coexpressing rCD2p110 and the GFP-tagged PKB-PH domain initiates a rapid relocation of the PKB construct from the plasma membrane into the cytosol and the nucleus. This striking response was complete within 30 s of adding the PI3K inhibitor to the B cell population (Fig. 6 B).

Coligation of the BCR with the FcγRIIb Prevents Membrane Localization of PKB

BCR activation of PKB is subject to a negative feedback control mechanism initiated by the FcγRIIB and mediated by SHIP (Fig. 1) (Aman et al., 1998). The stimulation of PKB catalytic function requires membrane localization of the kinase (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997) plus its phosphorylation at two residues: threonine 308 and serine 473 by PI3K-dependent protein ki-
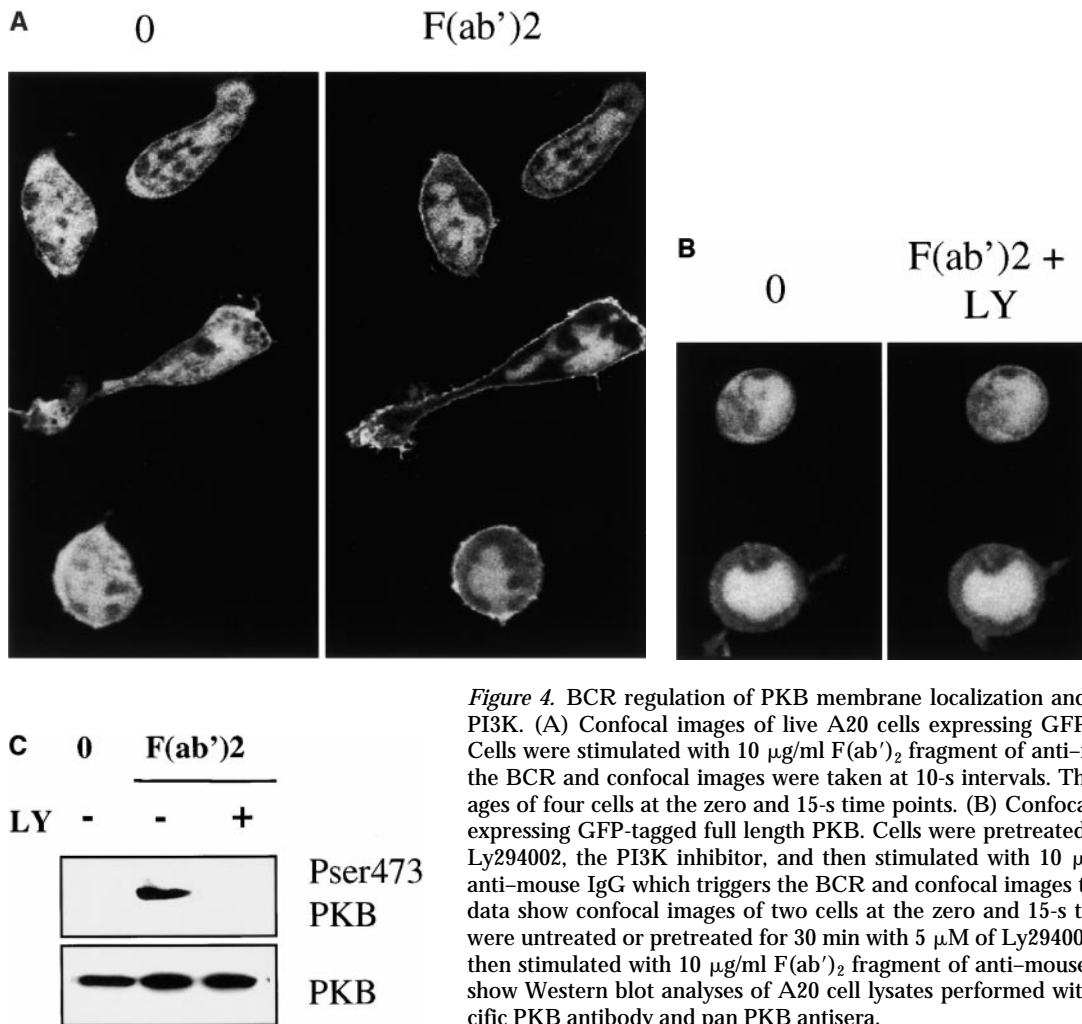
Figure 4. BCR regulation of PKB membrane localization and activity is dependent on PI3K. (A) Confocal images of live A20 cells expressing GFP-tagged full length PKB. Cells were stimulated with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR and confocal images were taken at 10-s intervals. The data show confocal images of four cells at the zero and 15-s time points. (B) Confocal images of live A20 cells expressing GFP-tagged full length PKB. Cells were pretreated for 30 min with 5 μM of Ly294002, the PI3K inhibitor, and then stimulated with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR and confocal images taken at 5-s intervals. The data show confocal images of two cells at the zero and 15-s time points. (C) A20 cells were untreated or pretreated for 30 min with 5 μM of Ly294002, the PI3K inhibitor, and then stimulated with 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG for 5 min. The data show Western blot analyses of A20 cell lysates performed with serine 473 phosphospecific PKB antibody and pan PKB antisera.

nases (Alessi et al., 1997; Alessi and Cohen, 1998; Bellacosa et al., 1998; Stephens et al., 1998). The FcγRIIB/SHIP complex will influence both the kinetics and magnitude of PIP3 levels during B cell activation and could remove a membrane targeting signal for PKB. However, SHIP dephosphorylates PI(3,4,5)P$_3$ to form PI(3,4)P$_2$ which is also able to bind to the PH domain of PKB (Franke et al., 1997; Frech et al., 1997). Thus, it is possible that PKB will still be recruited to the plasma membrane when the BCR is coligated with the FcγRIIB complex and that SHIP terminates PKB-mediated responses by preventing the phosphorylation and activation of membrane-localized PKB. For example, osmotic stress can prevent PKB activation without preventing membrane localization of Akt/PKB (Meier et al., 1998). To study the effects of BCR coligation with the FcγRIIB on the cellular localization of PKB, A20 cells expressing the GFP-PKB constructs were stimulated with either F(ab')$_2$ fragment of anti–mouse IgG or with intact IgG. Confocal images of a midsection of the cells were then taken at 5-s intervals after addition of the stimulus. The results in Fig. 7 show that B cell activation with intact IgG, which cross-links the BCR with the FcγRIIB, fails to stimulate PKB membrane translocation. Fig. 7 shows confocal images of cells 15 s after BCR triggering but cells

were monitored for several minutes and no membrane localization of PKB could be seen in BCR/FcγRIIB coligated cells at any time point. In the presence of an anti-FcγRIIB antibody which blocks the interaction of IgG with the FcγRIIB receptor, intact IgG is able to trigger the BCR without coligating inhibitory FcγRIIB into the BCR complex. The results show that presence of the blocking antibody to the FcγRIIB then allows intact IgG to induce plasma membrane localization of PKB.

Discussion

This study has used GFP-tagged PKB and time lapse confocal microscopy of live cells to follow the cellular localization of PKB during antigen receptor activation of B lymphocytes. The data show that BCR regulation of PKB is a dynamic process; there is an initial rapid and transient recruitment of PKB to the plasma membrane followed by a sustained activation of the enzyme. PKB localization to the plasma membrane can be detected within 10 s of BCR triggering but the response has finished after a further 40–50 s. Stimulation of PKB catalytic activity is similarly rapid and can be detected within 30 s of BCR triggering but the activation of PKB is maintained for at least 1 h after BCR
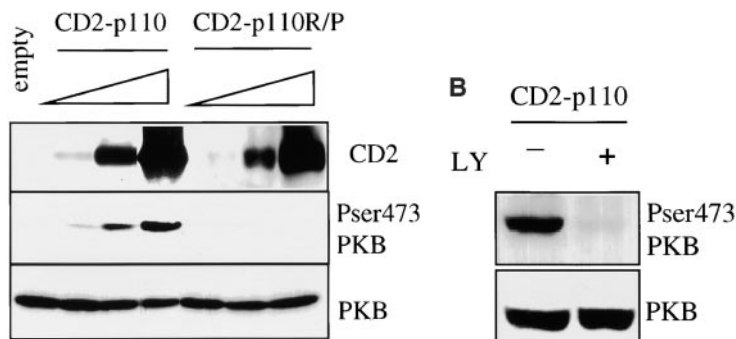
Figure 5. PI3K signals are sufficient to activate PKB in B cells. (A) A20 cells were transfected with either 5, 10, or 20 μg of pEF rCD2p110 (active PI3K) or the equivalent amounts of the control catalytically inactive pEF rCD2p110R/P mutant. Cells were maintained in culture for 14 h. The data show Western blot analysis of total cell lysates from control cells or cells expressing the PI3K mutant. The top shows Western blot experiments with the rat CD2 antibody OX34 which monitors cellular levels of the rCD2 PI3K chimeras. The middle and bottom show, respectively, Western blot analyses with serine 473 phosphospecific PKB antibody and pan PKB antisera. (B) A20 cells were transfected with 20 μg of pEF rCD2p110 (active PI3K). Cells were maintained in culture for 14 h. The data show Western blot analysis of total cell lysates from cells expressing rCD2p110 or after 3 h of incubation with 5 μM of Ly294002. The data show Western blot analyses with serine 473 phosphospecific PKB antibody (top) and pan PKB antisera (bottom).

triggering. PKB is distributed throughout the cytosol and the nucleus of both quiescent and BCR-triggered B cells. More importantly, in BCR-triggered B cells, phosphorylated active PKB is found in both the cytosol and nucleus. The question of the localization of active PKB is important because it affords insight as to the potential site of action of this kinase during B cell activation. The present results show that PKB has three potential sites of action during B cell activation. Transiently after BCR triggering, PKB could function to phosphorylate plasma membrane localized targets but during the sustained B cell response PKB is acting in the nucleus and the cytosol. PKB is always present in the nucleus of the A20 cells but we have no way to test whether the PKB that one sees in the nu-
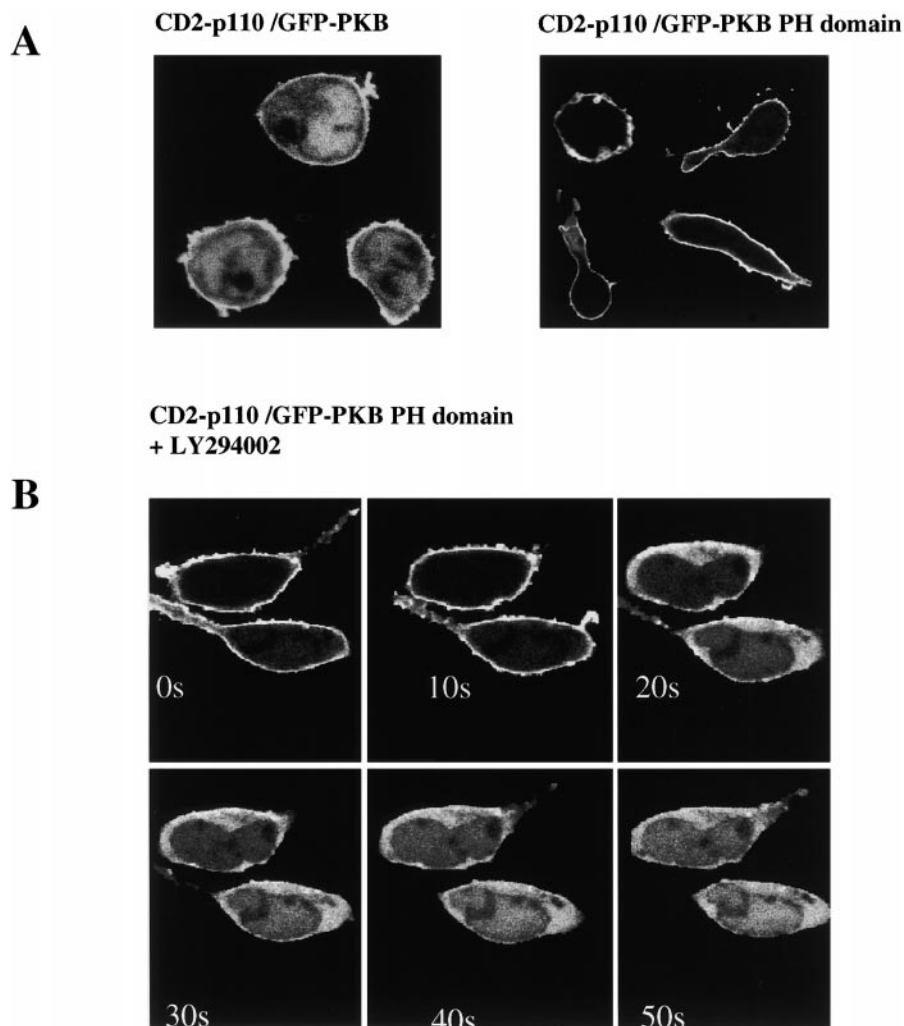

Figure 6. PKB localization in cells expressing constitutively active PI3K. (A) Confocal images of live A20 cells cotransfected with 20 μg of pEF rCD2p110 (active PI3K) and either GFP-tagged full length PKB (left) or GFP-tagged PKB-PH domain (right). After transfection, cells were maintained in culture for 14 h before confocal imaging. (B) Confocal images of live A20 cells cotransfected with 20 μg of pEF rCD2p110 (active PI3K) and GFP-tagged PKB-PH domain. After transfection, cells were maintained in culture for 14 h before confocal imaging. Confocal images were taken at 10-s intervals after addition of 5 μM of Ly294002 to inhibit PI3K activity.
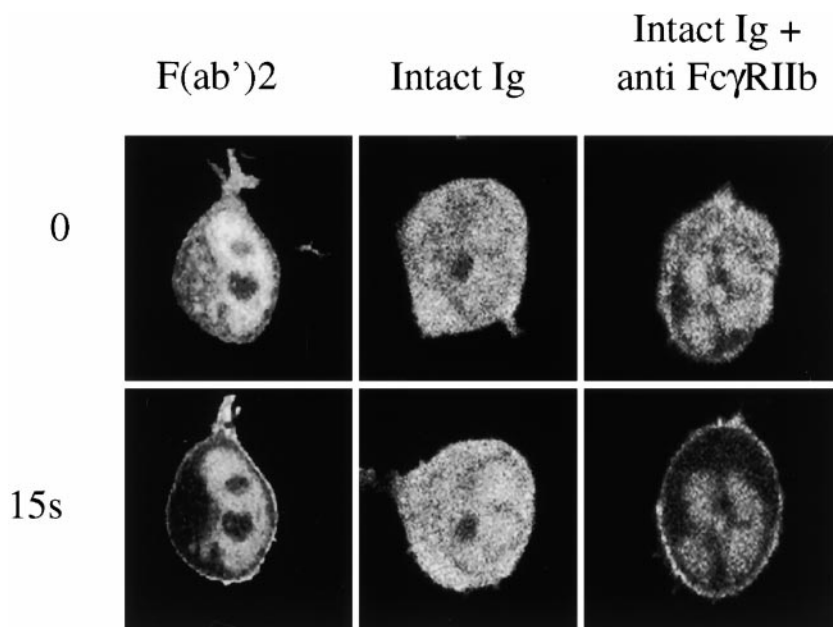
F(ab')2   Intact Ig   Intact Ig +
                        anti FcγRIIb

0

15s

Figure 7. The FcγRIIb prevents membrane translocation of PKB. Confocal images of live A20 cells expressing GFP-tagged full length PKB. A20 cells stimulated at 37°C with the following stimuli: 10 μg/ml F(ab')$_2$ fragment of anti–mouse IgG which triggers the BCR; 15 μg/ml of intact Ig which coligates the BCR and the FcγRIIB complex; and 15 μg/ml of intact Ig in cells pretreated for 30 min with 2.5 μg/ml anti-FcγRIIB which prevents FcγRIIB/BCR coligation and allows intact Ig to trigger the BCR. Confocal images were taken at 5-s intervals; the data show confocal images taken 15 s after A20 cell stimulation.

cleus at time zero is the same PKB found in the nucleus several minutes after BCR triggering. Mutations in the PKB-PH domain that prevent PKB plasma membrane targeting prevent PKB activation and we therefore assume that the active PKB that we see in the nucleus has been activated at the membrane. This would mean that there is a dynamic turnover from the cytosol/nucleus to the membrane and back to the cytosol and nucleus. The present results show that this cycle could be completed within 30–40 s. The results in Fig. 2 show that PKB can move to the plasma membrane within 10 s of activating the BCR. The data in Fig. 6 show that the PKB-PH domain can leave the membrane and enter the nucleus within 20–30 s. So PKB could do a full cycle, cytosol/plasma membrane/nucleus, within 30–40 s.

The FcγRIIb mediates vital homeostatic control of B cell function by recruiting an inositol 5 phosphatase, SHIP, into the BCR complex. The importance of the FcγRIIB/SHIP complex for B cell homeostasis is illustrated by the phenotype of mice lacking expression of either FcγRIIB or SHIP which are prone to inflammatory disease and show a higher sensitivity to anaphylaxis (Takai et al., 1996; Helgason et al., 1998). FcγRIIB ligation with the BCR prevents activation of PKB; this response was revealed by quantitation of PKB activity in vitro but was confirmed also by analysis of the effects of BCR and FcγRIIB coligation on the phosphorylation of GSK3, an endogenous substrate for PKB. SHIP dephosphorylates PI(3,4,5)P$_3$ to produce PI(3,4)P$_2$ and is essential for the inhibitory action of the FcγRIIB (Ono et al., 1997). The PH domain of PKB, which mediates membrane targeting of the enzyme, is able to bind the product of SHIP, PI(3,4)P$_2$, in vitro. Nevertheless, the present data show that coligation of the BCR with the inhibitory FcγRIIB prevents membrane targeting of PKB. This was an intriguing result that reveals a mechanism for the inhibitory action of the FcγRIIB on the BCR/PKB response. It also suggests that binding of PI(3,4)P$_2$ to the PH domain of PKB in vivo is insufficient to target

PKB to the membrane. However, it should be emphasized that accumulation of PI(3,4)P$_2$ in BCR/FcγRIIb activated B cells and has not been formally shown.

The PKB-PH domain was recruited stably to the membrane in BCR-activated cells and also in cells expressing constitutively active PI3K. The PKB-PH domain binds to PIP3 and its translocation to the plasma membrane in B cells was absolutely dependent on the continued presence of D3 phosphoinositides: inhibition of the catalytic activity of PI3K with LY294002 causes immediate loss of the PKB-PH domain from the plasma membrane. It was particularly striking that within 30–40 s of adding the PI3K inhibitor, the PKB-PH domain moves from the plasma membrane into the cytosol and the nucleus of the B cell. The stability of the membrane localization of the PKB-PH domain was in marked contrast to the transient membrane residence of intact wild-type PKB under conditions of continuous PI3K activation or BCR triggering. The loss of wild-type active PKB from the plasma membrane within 1 min of BCR ligation is intriguing and cannot be explained by limits in levels of cellular PIP3 because PIP3 levels are elevated in A20 cells for at least 15 min after BCR ligation (data not shown). Moreover, PKB is not maintained at the membrane in cells expressing constitutively active PI3K although the stability of the membrane targeting of the PKB-PH domain confirms that there are sufficient levels of D-3 phosphoinositides in activated B cells to tether PKB to the membrane. Previous models have suggested that PKB colocalizes with active PI3K to membrane sites with elevated levels of D-3 phosphoinositides. The present confocal imaging data comparing the localization of full length PKB and the PKB-PH domain in B cells show that the PKB-PH domain follows this model but the full length PKB molecule does not. These studies thus reveal the existence of a molecular mechanism that must cause active PKB to dissociate from the plasma membrane in B cells despite the continued generation of PIP3. One possibility is that phosphorylated and/or active full length PKB un-

dergoes a conformational change that prevents continued lipid binding. Alternatively, PKB interactions with substrate may relocalize the active kinase to the cytosol and nucleus.

In summary, this study has used confocal imaging coupled with more classical biochemical analyses to study the dynamic PI3K-regulated processes that occur during B cell activation. The BCR triggers a transient membrane localization of PKB but a sustained activation of the enzyme; active PKB is found in the cytosol and nuclei of BCR-stimulated B cells. This result affords new information as to the potential site of action of PKB during B cell activation. Recent genetic studies have shown that PI3K is important for B cell development and for B cell function in the peripheral lymphoid compartment. The present results show that PI3K signals are both necessary and sufficient for sustained activation of PKB in B lymphocytes which firmly positions Akt/PKB in PI3K-mediated signaling pathways in B lymphocytes. There has been much recent information about the effects of antigen receptors on membrane-localized signaling pathways in lymphocytes; the significance of the present report is that the PI3K/PKB pathway couples signaling events triggered at the lymphocyte membrane to the cell nucleus.

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